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Title

Calibration of the comprehensive NDHA-N₂O dynamics model for nitrifier-enriched biomass using targeted respirometric assays.

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Abstract

The NDHA model comprehensively describes nitrous oxide (N₂O) producing pathways by both autotrophic ammonium oxidizing and heterotrophic bacteria. The model was calibrated via a set of targeted extant respirometric assays using enriched nitrifying biomass from a lab-scale reactor. Biomass response to ammonium, hydroxylamine, nitrite and N₂O additions under aerobic and anaerobic conditions were tracked with continuous measurement of dissolved oxygen (DO) and N₂O.

The sequential addition of substrate pulses allowed the isolation of oxygen-consuming processes. The parameters to be estimated were determined by the information content of the datasets using identifiability analysis. Dynamic DO profiles were used to calibrate five parameters corresponding to endogenous, nitrite oxidation and ammonium oxidation processes. The subsequent N₂O calibration was not significantly affected by the uncertainty propagated from the DO calibration because of the high accuracy of the estimates. Five parameters describing the individual contribution of three biological N₂O pathways were estimated accurately (variance/mean < 10% for all estimated parameters).

The NDHA model response was evaluated with statistical metrics (F-test, autocorrelation function). The 95% confidence intervals of DO and N₂O predictions based on the uncertainty obtained during calibration are studied for the first time. The measured data fall within the 95% confidence interval of the predictions, indicating a good model description. Overall, accurate parameter estimation and identifiability analysis of ammonium removal significantly decreases the uncertainty propagated to N₂O production, which is expected to benefit N₂O model discrimination studies and reliable full scale applications.

Keywords: Nitrous oxide, Respirometry, Uncertainty, Modelling, Parameter Estimation

1. Introduction

Nitrous oxide (N_2O) is a greenhouse gas emitted during biological nitrogen removal (BNR). The carbon footprint of wastewater treatment plants (WWTPs) is highly sensitive to N_2O emissions (Gustavsson and Tumlin, 2013), thus reducing N_2O emissions is beneficial for the energy balance of WWTPs.

During BNR operations biological and abiotic pathways are responsible of N_2O emissions (Schreiber et al., 2012). Ammonia-oxidizing bacteria (AOB) produce N_2O during incomplete ammonium (NH_4^+) oxidation to nitrite (NO_2^-) (nitrifier nitrification, NN). Under low dissolved oxygen (DO) AOBs use NO_2^- as the terminal electron acceptor and also release N_2O (nitrifier denitrification, ND). Heterotrophic denitrification is a 4-step process where N_2O is an obligate intermediate. Under low carbon-to-nitrogen ratios or in the presence of DO heterotrophic denitrification is not complete and N_2O can be released (HD) (Richardson et al., 2009). Hydroxylamine (NH_2OH) and free nitrous acid (HNO_2) are intermediates of NH_4^+ oxidation which can produce N_2O abiotically.

Process models are useful tools that translate our understanding of N_2O production into mathematical equations. N_2O model structures vary depending on the number of pathways, nitrogenous variables or parameters considered (Ding et al., 2016; Ni et al., 2014; Pocquet et al., 2016). The description of the autotrophic contribution transitioned from single- (NN or ND) to two-pathway (NN and ND) models to capture the N_2O dynamics observed during N-removal (Pocquet et al., 2016; Schreiber et al., 2009). Recently, a consistent N_2O model was proposed (NDHA, Nitrifier nitrification, nitrifier Denitrification, Heterotrophic denitrification and Abiotic) that predict three biological pathways and abiotic processes (Domingo-Félez and Smets, 2016). Potentially, the NDHA model describes N_2O production under a wide range of operational conditions.

N_2O models are extensions of existing structures describing nitrogen removal and thus, calibration of N_2O dynamics also requires accurate predictions of the primary substrates (i.e. DO, NH_4^+ , NO_2^-

, etc.). The experimental datasets used for calibration in lab-scale systems are either directly obtained from the reactor performance (Ding et al., 2016) or by conducting batch experiments (Ni et al., 2011). Initially, the information content of the experimental design was not studied because models aimed at describing N₂O trends without focusing on rigorous calibrations (Law et al., 2011). However, the amount and quality of data of the experimental design directly impact the calibration results (Dochain and Vanrolleghem, 2001).

Some studies report the proposed calibration framework (Guo and Vanrolleghem, 2014), but the N₂O parameter estimation procedures are often ill-described, with little information about each step. For example, the parameter subset selection considered during parameter estimation is sometimes not addressed. Local sensitivity measures are used as rankings for parameter selection (Pocquet et al., 2016; Spérandio et al., 2016), but these rankings are dependent on the initial parameter values and do not capture parameter interactions (Brun et al., 2001).

The overall fit and capabilities to describe N₂O dynamics has relied on analysis from best-fit simulations (e.g. R²), which can lead to ambiguous results that cannot discriminate between models (Lang et al., 2017; Pan et al., 2015). A more rigorous analysis of residuals (e.g. Gaussian distributions, autocorrelation functions (ACF), F-test, etc.) would benefit the validation of the model response (Bennett et al., 2013).

Also, addressing the practical identifiability of newly estimated parameters will improve N₂O model discriminations procedures. For example, the parameter variance and correlation matrix are indicators of the confidence that can be given to a value, but they are not always reported, which makes it difficult to compare between N₂O model predictions (Ding et al., 2016; Kim et al., 2017; Pocquet et al., 2016; Spérandio et al., 2016). Practical identifiability problems might contribute to the large variability of parameter values in N₂O models (Domingo-Félez et al., 2017).

The uncertainty obtained during calibration translates into confidence intervals for model predictions. The accuracy, or width of the confidence interval, associated to the N₂O emissions

will be a key factor to consider during the development of mitigation strategies. Yet, the uncertainty of N₂O emissions associated to model calibration is not studied.

The objective of this study is to demonstrate and evaluate a standardized procedure for parameter estimation from N₂O models that relies on respirometric assays and in particular its application to analyse and validate the recently developed NDHA models. These assays are designed to allow the sequential fit of model components. The novelty resides in improving N₂O calibration procedures by targeting sources of uncertainty. Subsequently, the calibration results and associated uncertainty are evaluated. The calibration approach presented is a rigorous tool beneficial for N₂O model discrimination.

2. Material and Methods

2.1. Experimental Design

Nitrifying enrichment culture.

A lab-scale nitrifying sequencing batch reactor (5 L) was operated and displayed stable performance for three months after enrichment from an AS mixed liquor sample. Synthetic wastewater (modified after Graaf et al., 1996) with NH_4^+ as the only nutrient was fed at 0.5 g NH_4^+ -N/L·d and a constant aeration rate maintained oxygen-limited conditions (DO below 0.25 mg/L) (SI-S1). NH_4^+ removal was $82 \pm 14\%$, and nitrification efficiency ($\text{NO}_2^-/\text{NH}_4^+$ removed) at $85 \pm 24\%$ (Su et al., 2017). The biomass composition, based on 16 rRNA targeted qPCR analysis had a dominance of AOB over NOB (30:1). Detailed information of the qPCR analysis can be found in (Terada et al., 2010).

Monitoring nitrification and N_2O production via extant respirometric assays.

Biomass samples were harvested towards the end of the react cycle by centrifugation at 3600 g for 5 min, washed and resuspended in nitrogen-free mineral medium three times to eliminate any soluble substrate.

Assays were performed in parallel at 25°C in two 400-mL jacketed glass vessels completely filled with biomass and sealed with the insertion of a Clark-type polarographic DO electrode (YSI Model 5331, Yellow Springs, OH). Biomass samples were saturated with air or pure oxygen prior to the initiation of the respirometric assays. A decrease in the DO level in the vessel due to substrate oxidation was measured by the DO probe and continuously acquired by a personal computer interfaced to a DO monitor (YSI Model 5300, Yellow Springs, OH) by a multi-channel data acquisition device (LabPC+, National Instruments, Austin, TX). DO profiles were acquired at a user-defined frequency below the response time of the sensor (0.2 Hz). Liquid N_2O concentrations were measured with Clark-type microsensors (N2O-R, Unisense A/S, Aarhus, Denmark) and pH (WTW GmbH, Weilheim, Germany). Stock solutions for all the reagents were

prepared from high-purity chemicals for NH_4HCO_3 , $\text{NH}_2\text{OH}\cdot\text{HCl}$, NaNO_2 , $\text{C}_3\text{H}_5\text{NaO}_2$ (Sigma Aldrich) and by sparging $\geq 99.998\%$ gas in deionized water for N_2O (Sigma-Aldrich). Photometric test kits were used to analyze N-substrates (1.14752, 1.09713, 1.14776, Merck KGaA, Darmstadt, Germany).

[Table 1]

Experimental Design

The aim of the experimental design was to obtain informative data on N_2O dynamics for a nitrifier dominated biomass to allow estimation of parameters of the NDHA model, which captures processes associated with nitrification, denitrification, and abiotic processes. Respirometric approaches were exclusively taken (on-line, high-rate O_2 and N_2O measurements) as they allow accurate parameter estimates compared to substrate depletion experiments (Chandran et al., 2008). The kinetics of the oxidation of the primary N-substrates (NH_4^+ , NH_2OH and NO_2^-) were individually and step-wise, measured via extant respirometry under various initial DO conditions, with continuous N_2O measurements (Table 1). Then the interaction between the different N species was ascertained by liquid sampling at the end of certain experiments. In addition, specific experiments were conducted to measure the heterotrophic and abiotic contributions to total N_2O production during nitrification. Biomass content (MLSS, MLVSS) was measured in duplicates according to APHA (APHA et al., 1999).

The purpose was to predict the fate of the primary N-substrates based on the specific oxygen-consuming rate. By sequentially adding substrate pulses from oxidized to reduced form ($\text{NO}_2^- \rightarrow \text{NH}_2\text{OH} \rightarrow \text{NH}_4^+$), the individual rates can be isolated (Brouwer et al., 1998).. The N_2O dynamics from NH_4^+ removal at varying NO_2^- and DO concentrations can be investigated simultaneously.

Scenarios (Batches grouped by substrate added: NH_4^+ , NH_2OH , NO_2^-)

A scenario (e.g. Scen_AMO) was defined as a group of experiments with the same primary N-substrate added by pulses (Table 1). The overall oxygen consumption was the additive effect of

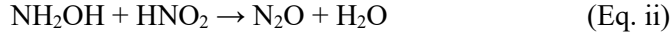
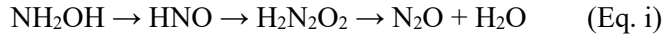
several independent oxygen consumption processes, potentially including endogenous -, NO_2^- -, NH_2OH -, and NH_4^+ -respiration.

2.2. N_2O model description: NDHA

The NDHA model was proposed as a consistent model to describe N_2O dynamics under a variety of conditions for biomass containing both autotrophic and heterotrophic fractions (Domingo-Félez and Smets, 2016). It considers N_2O production from two autotrophic and one heterotrophic biological pathways, plus abiotic N_2O formation based on recent findings (Soler-Jofra et al., 2016). Unlike any other model, NDHA can qualitatively capture NO and N_2O profiles that have been observed at high and low DO (Castro-Barros et al., 2016; Kampschreur et al., 2008; Rodriguez-Caballero and Pijuan, 2013; Yu et al., 2010). Here we aim to calibrate the NDHA model.

The following summarizes the essential and unique components of NDHA; for more information see (Domingo-Félez and Smets, 2016). The two autotrophic pathways have two different NO-producing processes, which are combined into a single N_2O -producing process. In Nitrifier Nitrification (NN), NO_{NN} is produced during NH_4^+ oxidation (AOR) under oxic conditions. Higher AOR will likely increase NO_{NN} and also N_2O . A fraction of NH_4^+ , proportional to AOR is always released as $\text{N}_2\text{O}_{\text{NN}}$. In autotrophic denitrification (ND), under low DO NO_{ND} is produced by the reduction of HNO_2 with NH_2OH . This step is negatively affected by DO. The reduction of both NO_{ND} and NO_{NN} is lumped in one process with no oxygen inhibition as it is not known whether both NIR and NOR steps are directly inhibited by DO (Kozłowski et al., 2014). Thus, if NIR is inhibited by DO the overall ND-associated N_2O production will be indirectly limited. The 4-step denitrification model was considered based on (Hiatt and Grady, 2008). Individual process rates and inhibition/substrate coefficients were used as suggested for systems with low substrate accumulation. Nitrification produces HNO_2 and NH_2OH . Abiotically NH_2OH can form HNO which dimerizes via $\text{H}_2\text{N}_2\text{O}_2$ to N_2O and H_2O (Eq. i). HNO accumulation could occur due to an imbalance between the two reactions, leading to chemical N_2O production (Igarashi et al., 1997).

Nitrosation of NH_2OH (Eq. ii) has also been postulated as a relevant reaction in partial nitrification reactors (Soler-Jofra et al., 2016).



The pH data was used as input to the model to calculate the corresponding NH_3 and HNO_2 concentrations.

2.3. Parameter estimation procedure

The steps in the parameter estimation procedure were to (1) estimate the best fit parameters to describe O_2 consumption and N_2O production during the various (or during each type of) experimental scenarios (Figure 1), (2) estimate the contribution of separate pathways to the total N_2O production, and (3) quantify the uncertainty of model predictions.

[Figure 1]

2.3.1. Parameter subset selection - Global sensitivity analysis

A global sensitivity analysis (GSA) was performed to identify the parameters most determinant of model outputs by linear regression of Monte-Carlo simulations (Sin et al., 2009). Uncertainty from model parameters was propagated as 10-25-50% uniform variations from their default value to model outputs. Latin hypercube sampling was used to cover the parameter space. The Standardized Regression Coefficient method was used to calculate the sensitivity measure β_i , which indicates the effect of the parameter on the corresponding model output (Campolongo and Saltelli, 1997) (convergence found with 500 samples). The duration of every experiment was discretized in 400 parts and the GSA run at each point (SI-S2).

Parameters describing the elemental biomass composition (e.g. i_{NXB}), yield and temperature coefficients were fixed at default values and not considered for calibration. For each scenario the top ranked most sensitive parameters were preliminary selected as candidates for parameter estimation. All possible combinations of parameter candidates were assessed by increasing the

size of the calibration subset to find the largest identifiable subset with the lowest error, assessed by the Akaike Information Criterion (AIC) (Akaike, 1974). To compare the information content of different parameter subsets of the same size the optimal experimental design criteria mode (Dochain and Vanrolleghem, 2001) was calculated together with RDE (Ratio of normalized D to modified E criteria), which captures the accuracy and precision of a calibrated subset (Machado et al., 2009). Newly estimated parameters were fixed at their best-fit estimate on the next calibration step (Figure 1).

2.3.2. Error minimization

The error function for problem minimization was defined as:

$$\text{Error} = \sum_j^m \frac{1}{n} \sum_i^n \left(\frac{y_{sim,i} - y_{obs,i}}{\sigma_i} \right)^2$$

Where m is the number of experiments in one scenario (e.g. 2 NOB experiments in Scen_NOB), n the number of experimental points of each experiment, $y_{sim,i}$ the model prediction and $y_{obs,i}$ the experimental data at time i , and σ_i the standard error of the experimental data. The minimization problem was started with global search method over a wide parameter space (GlobalSearch algorithm). From the estimated minimum, multiple local searches (PatternSearch algorithm) were started randomly in a narrower parameter space to avoid local minima. Model simulations were performed in the Matlab environment (The Mathworks Inc., Natick, USA).

2.3.3. Validation of model response and parameter estimates

To test the validity of the model response (i.e. the adequacy of model to predict the observed data points) the residuals ($y_{sim,i} - y_{obs,i}$) were compared to a Gaussian distribution with a one-sample Kolmogorov-Smirnov test (Lilliefors, 1967). Interdependency of residuals was analysed by autocorrelation for different lag times (Cierkens et al., 2012). The quality of the model fit was calculated via correlation coefficients (R^2) and challenging the hypothesis of the linear regression with simultaneous unit slope and zero intercept, where a value of 0/1 indicates a bad/good model fit (F-test). Moreover, by separating the error into three components: means, slope differences

and randomness the Mean Squared Error Prediction (MSEP) index identifies the main error source between randomness, mean and standard deviation of residuals (NC, ME, SE) (Haefner, 2005). The prediction accuracy and the validation of the model to individual experiments was evaluated by the Root Mean Squared Error (RMSE) and the Janus coefficient to compare the RMSE between model calibration and validation (Power, 1993).

Based on the Fisher Information Matrix (FIM) collinearity indices were calculated to evaluate the identifiability of parameter estimates (Brun et al., 2001). Approximate confidence regions were calculated following $J_{crit} = J_{opt} \left(1 + \frac{p}{N_{data}-p} F_{\alpha,p,N_{data}-p} \right)$ (Beale, 1960). Coefficients of variation (CV) were described as the ration between the variance (σ) and the mean (μ) of the estimate.

The reliability of predictive distributions (95% confidence intervals) was evaluated by calculating the Percentage of observations within the Unit Confidence Interval (PUCI) (Li et al., 2011), which combines the fraction of experimental points inside the confidence interval (PCI) and the Average Relative Interval Length (ARIL) $ARIL_{0.95} = \overline{(\text{Limit}_{Upper,0.95} - \text{Limit}_{Lower,0.95})} / \text{Data}$ (Jin et al., 2010). A smaller ARIL value (narrow distance between upper and lower 95% CI predictions) and a larger PUCI represent a better performance.

2.4. Uncertainty evaluation

The effect of directly estimated versus assumed parameter uncertainty was evaluated by Monte-Carlo simulations. The parameter distribution was sampled via LHS (n = 500) for two cases: from the distributions obtained during calibration, and compared to uncertainty classes assumed from literature as a reference case (Sin et al., 2009). The resolution of prediction uncertainty was assessed by the ARIL.

3. Results

3.1. Oxygen consumption during respirometric assays.

Each scenario grouped experiments based on the substrate added: NO_2^- (Scen_NOB) NH_2OH (Scen_HAO) or NH_4^+ (Scen_AMO). In all scenarios, even prior to any substrate spikes, oxygen consumption was always positive and proportional to the biomass concentration due to endogenous respiration. After substrate addition, oxygen consumption increased, to a much higher rate with NH_4^+ or NH_2OH than with NO_2^- spikes (Figure 2). The lower NO_2^- oxidizing rate of the biomass agreed with the measured low NOB vs AOB abundance (ca. 1:30). pH was not controlled and varied between 7 and 8, decreasing during NH_4^+ oxidation and increasing during reaeration periods due to CO_2 stripping or by manual base addition (NaOH).

[Figure 2]

The ranking from the averaged GSA for DO shows that the sequential scenarios (measuring the respirometric response to addition of synthetic substrates) provides sufficient information to individually estimate the relevant biokinetic parameters of each step in the ammonium oxidation process (Figure 3). For example, in Scen_NOB the maximum growth rate for NOB (μ_{NOB}) ranked first, while in Scen_AMO the substrate affinity ($K_{\text{AOB.NH}_3}$) and maximum growth rate for the AMO ($\mu_{\text{AOB.AMO}}$) step ranked in the top.

[Figure 3]

3.1.1. Estimation of primary substrate kinetics based on DO profiles

Initial conditions for each batch were defined by simulating endogenous decay and hydrolysis with default parameters. The objective of the parameter subset selection was to calibrate the minimum number of identifiable parameters that explain the data. A total of five parameters could be estimated from the aerobic scenarios (Table 2). The calibrated parameters for Scen_NOB were μ_{NOB} and k_{H} , for Scen_AMO μ_{AMO} and $K_{\text{AOB.NH}_3}$, and for Scen_AMO_DO $K_{\text{AOB.O}_2.\text{AMO}}$ (Table 2). To illustrate the procedure used for every scenario results from Scen_NOB and Scen_AMO are

summarized in the SI (SI-S3). The electron distribution in AOB differs between NH_4^+ (Scen_AMO) oxidation and isolated NH_2OH oxidation (Scen_HAO) (SI-S4). Hence, parameter estimation results from Scen_HAO were not considered representative of NH_4^+ oxidation, our targeted process. To describe more accurately the low NH_2OH concentration values reported in literature (Soler-Jofra et al., 2016) the affinity for NH_2OH , $K_{\text{AOB.NH}_2\text{OH}}$, was increased compared to other N_2O models (SI-S4). The oxygen consumption rate of NH_4^+ experiments drops quickly to endogenous levels (Figure 2, C). The lack of an intermediate oxygen uptake rate indicates that no significant accumulation of an intermediate which agrees with the proposed higher affinity for NH_2OH (2.29 mgCOD/mgN). [Table 2]

Validation of model response (DO) and primary N-substrate parameter estimates

The model consistently described the experimental DO profiles for every scenario (F-test = 1, which indicates that we fail to reject the null hypothesis of slope 1 and intercept 0 between simulations and observations) (Figure 4, A, B). The MSEP indicated that randomness was the main source of error compared to the mean or standard deviation, validating the model response during calibration (NC > ME, SE, SI-S5). The uncertainty of the parameter estimates (Table 2) was propagated to the model predictions, showing an increased resolution of the 95% predictive distributions for DO compared to the uncertainty of the reference case (ARIL = 3.8/0.5 before/after parameter estimation). The PUCI (percentage of observations bracketed by the unit confidence interval) also improved from 0.4 to 1.5.

Best-fit parameter estimates at each scenario were estimated at high accuracy: coefficients of variation (CV) were below 7% for all cases (Table 2) and the collinearity indices below 15, as suggested for identifiable subsets (Brun et al., 2002) (SI-S3). The high correlation between $\mu_{\text{AOB.AMO}}\text{-}K_{\text{AOB.NH}_3}$ typically occurs for Monod-type kinetics but it did not affect their identifiability.

While the error distribution of each scenario was not normally distributed (Kolmogorov-Smirnov test 95%), no systematic deviations were observed (Figure 4). The analysis of the residuals

indicated that for scenarios AMO and NOB the errors were autocorrelated (SI-S5). Subsequently, the effect of sampling resolution on the optimal parameter values and uncertainties was minimized until the autocorrelation obtained was negligible (SI-S5). As the sampling data frequency decreased through subsampling, the accuracy of estimates decreased too (e.g. $CV_{\mu_{AOB,AMO}} = 2\%$ point/2 min, 4.4% point/10min). However, the lower precision of the best-fit estimates did not translate into higher simulation uncertainty for the primary N-substrates ($\sigma_{95\%CI}$ increased by less than 0.06 mg/L for DO, NH_4^+ , NH_2OH or NO_2^-). Consequently, while the autocorrelation of residuals affected the DO parameter estimation results it did not impact the N_2O calibration, the focus of this study.

[Figure 4]

The fitted model was evaluated on five additional experiments not used during calibration with varying initial pH (7-8), NO_2^- (0-6.2mgN/L) and NH_4^+ pulses (1-10mgN/L). The Janus coefficient and R^2 were close to unity (1.24 and 0.997) indicating a good model validation (Figure 4, C, D). In sum, the respirometric experimental design can be used to precisely identify and calibrate the primary substrate dynamics of the NDHA model based on the DO profiles.

3.2. Dynamics of N_2O during different scenarios

In the same scenarios considered for DO calibration liquid N_2O was also continuously measured (Figure 2). Moreover, the role of the primary N-substrates (NH_4^+ , NH_2OH , NO_2^- , and NO_3^-) on N_2O production was also studied under anoxic conditions (Table 1). Under conditions heterotrophic denitrification (Scen_An_HB) the presence of NO_2^- and NO_3^- did not show any net N_2O production (data not shown). However, N_2O was consumed after sCOD addition when no other N-substrate was present, indicating a positive HD contribution to the total N_2O pool (Figure 5). During NO_2^- oxidation experiments or at the onset of anoxia in the presence of NO_2^- and NO_3^- no N_2O production was detected (Figure 2, A).

AOB-driven NH_4^+ oxidation (Scen_AMO) produced a small amount of N_2O under aerobic conditions and significantly increased at the onset of anoxia (Figure 2). The specific N_2O production rate ($\text{mgN}_2\text{O-N/gVSS.min}$) obtained in duplicate experiments – carried out at varying biomass concentrations – were in close agreement, thus indicating biologically-driven N_2O production (SI-S6).

The role of NH_2OH as a direct precursor of N_2O was investigated in Scen_HAO. Under aerobic conditions, NH_2OH oxidation produced more N_2O than NH_4^+ oxidation. In addition, upon reaching anoxia the N_2O production rate also increased in the presence of NH_2OH (Figure 2, B). Under anoxic conditions (Scen_An_AOB) neither NH_4^+ nor NO_2^- produced N_2O individually (SI-S6). The spike of NH_2OH yielded the largest amount of N_2O specific to the amount of nitrogen spiked, and NO_2^- was not detected at the end of the experiment ($\text{NO}_2^- < 0.05 \text{ mgN/L}$). Hence, NH_2OH oxidation by AOB produces N_2O and does not require O_2 . The addition of an electron acceptor like NO_2^- to ongoing anoxic NH_2OH oxidation increased the net N_2O production rate, while addition of an electron donor as NH_4^+ did not (SI-S6).

Taken together, the N_2O production observed in all the scenarios can only be effectively predicted using the NDHA model compared to other N_2O models, especially the DO-independent N_2O production from NH_2OH oxidation and no NO_2^- production (Ding et al., 2016; Domingo-Félez and Smets, 2016; Ni et al., 2014; Pocquet et al., 2016).

3.2.1. Parameter estimation from N_2O dynamics

In the absence of stripping, heterotrophic denitrification is the only N_2O consuming process. First, the N_2O consumption potential of the biomass was estimated and the reduction factor was considered representative for the 4-step heterotrophic denitrification processes ($\eta_{\text{HD}} = 0.055$) (Figure 5, A). Then, N_2O production observed from NH_4^+ oxidation at high DO, where the interference of the two denitrifying pathways is minimum, was used to calibrate the NN pathway. ϵ_{AOB} , the most sensitive parameter at high DO was calibrated, $\epsilon_{\text{AOB}} = 0.00048$ (Figure 5, B, SI-S2). Experiments from Scen_AMO were designed to reach anoxia at varying HNO_2

concentrations (0.15-3 $\mu\text{gN/L}$). Parameters associated to the ND pathway were the most sensitive and were thus calibrated ($\eta_{\text{NOR}} = 0.16$, $K_{\text{AOB.HNO}_2} = 0.67 \text{ } \mu\text{gN/L}$, $K_{\text{NH}_2\text{OH.ND}} = 0.25 \text{ mgN/L}$) (SI-S2, S7). The abiotic contribution measured was low and not considered during parameter estimation (SI-S6). A total of five parameters could be estimated from the N_2O datasets of all the scenarios (Table 2).

[Figure 5]

Validation of model response and secondary substrate (N_2O) parameter estimates

The calibrated NDHA model described the N_2O production dynamics and yield observed in the calibration datasets (F-test = 1). In all but one of the assays randomness was the most important part of the error based on the MSEP analysis (SI-S5). After calibration the ARIL narrowed by 58% from the original resolution and the PUCI increased by 71% ($n = 6$ assays).

The predictive ability of the model was evaluated on three batches with lower HNO_2 and with higher NH_2OH pulses ($\text{HNO}_2 < 0.15 \text{ } \mu\text{gN/L}$, $\text{NH}_2\text{OH} = 2 \text{ mgN/L}$). The average Janus coefficient of the validation prediction was 1.57 and R^2 was 0.985, indicating a good validation (Figure 5, D, E, F). Hence, the NDHA model could describe the N_2O production rates at a range of DO and HNO_2 concentrations.

The simple experimental design allowed the isolation of the various components of N_2O dynamics during NH_4^+ oxidation, and the parameter estimation procedure the identification of relevant model parameters.

3.3. Model predictions under varying DO and HNO_2 : Scenario analysis

To investigate the effect of DO and HNO_2 on N_2O production the NDHA model was evaluated at varying DO and HNO_2 concentrations at $\text{pH} = 7.5$ (Figure 6) with the newly estimated parameters. The model predicted the largest N_2O emission at the lowest DO and high HNO_2 (> 20%, SI-S8); and the lowest N_2O emission at the highest DO and lowest HNO_2 (0.13%). The effect of increasing HNO_2 is seen at every DO level ($\text{DO}_{0.3}$: $0.33 \rightarrow 5.4\%$, $\text{DO}_{5.0}$: $0.13 \rightarrow 0.28\%$).

Conversely, increasing DO lowered the N₂O emission factor regardless of the HNO₂ level. The NO emission showed an increasing pattern with HNO₂ but a minimum was found at DO = 2.0 mg/L, further increasing at higher DO (SI-S8).

The contribution of the NN pathway was maximum when HNO₂ was not present and decreased with increasing HNO₂, at a faster rate at lower than at higher DO (2.4 and 47% respectively). The ND contribution followed opposite trends, indicating a shift between autotrophic pathways driven by HNO₂ and DO. The ND contribution increased with HNO₂, at a steeper rate at lower DO (97.4%) than at higher (53%). The HD contribution was maximum at low DO and high HNO₂ but only reached 0.2% (SI-S8).

[Figure 6]

4. Discussion

4.1. Parameter estimation from respirometric assays: oxygen consumption

The respirometric experiments were used to investigate the oxygen-consuming processes driven by the AOB-enriched biomass in the presence of reduced N-species (NH_4^+ , NH_2OH and NO_2^-). If a model captures accurately the relevant oxygen-consuming processes, then DO and the primary N-substrates are predicted accurately. The experimental design based on the concatenated oxygen consumption allowed the isolation of individual processes independently (endogenous $\rightarrow \text{NO}_2^- \rightarrow \text{NH}_4^+$) (Chandran and Smets, 2005).

The calibrated model could describe the endogenous oxygen uptake and NO_2^- oxidation in Scen_NOB. However, because of the low NOB abundance the oxygen consumption from NO_2^- oxidation was low, shown by a similar sensitivity of NOB and endogenous parameters to oxygen consumption after NO_2^- spikes (Figure 3).

In Scen_AMO oxygen consumption was very sensitive to NH_4^+ dynamics (SI-S2), which yielded precise estimates for $\mu_{\text{AOB.AMO}}$, $K_{\text{AOB.NH}_3}$ and $K_{\text{AOB.O}_2\text{AMO}}$ (Table 2). The maximum AOB growth rate ($\mu_{\text{AOB.AMO}} = 0.49 \text{ 1/d}$) is in the low range of literature values found for *N. europaea* (0.56-1.62 1/d) (Brockmann et al., 2008). The biomass concentration (X_{AOB}), growth yield (Y_{AOB}) and maximum growth rate cannot be simultaneously identified from short experiments solely with DO data (Ellis et al., 1996; Petersen et al., 2001). Hence, the estimated growth rate is linearly dependent on the fixed values for X_{AOB} and Y_{AOB} : a lower initial condition for X_{AOB} would yield a higher estimate for $\mu_{\text{AOB.AMO}}$. Overall, the maximum specific NH_4^+ oxidation, $7.54 \pm 0.1 \text{E-05 gN/gVSS}_{(\text{AOB})}/\text{h}$, was similar to other literature values for an AOB-enriched biomass (Ciudad et al., 2006). For the same NH_4^+ concentration, the higher oxygen consumption rate observed at higher pH was predicted by considering NH_3 the true substrate. The estimated affinities for both NH_4^+ oxidation substrates ($K_{\text{AOB.NH}_3} = 0.12 \text{ mgN/L}$, $K_{\text{AOB.O}_2\text{AMO}} = 0.23 \text{ mg/L}$) were in range of literature values (Magrí et al., 2007; Park and Noguera, 2007).

Overall, the precision of the identified parameter was high ($\text{CV} < 7\%$), common from respirometric studies (Petersen et al., 2001). It should be noted that the concentration of the spikes

did not include uncertainty and was not estimated, which decreased the uncertainty of model predictions (Gernaey et al., 2002).

4.2. Role of NH_4^+ oxidation intermediates on N_2O production: experimental and modelling results

Nitrification plays an important role on N_2O emissions from N-removing systems, where NH_4^+ , NO_2^- and DO are the main substrates. Experimental results indicated that aerobic NH_4^+ -oxidation products, NH_2OH and NO_2^- were responsible for the higher N_2O production rate at the onset of anoxia and not NH_4^+ itself, which requires molecular O_2 for its oxidation (Sayavedra-Soto et al., 1996) (SI-S6). NH_2OH has been shown to be a key compound regulating N_2O production by AOB (Caranto et al., 2016; de Bruijn et al., 1995; Kozłowski et al., 2016). Because of its high reactivity under aerobic and anoxic conditions, it is an important electron donor for the cytochrome pool of AOB. Previous studies have shown the higher N_2O yield of nitrifying biomass and pure cultures fed on NH_2OH compared to NH_4^+ , also observed in Scen_HAO ($\text{N}_2\text{O_R}_{\text{NH}_2\text{OH}}/\text{N}_2\text{O_R}_{\text{NH}_4^+} = 40$) (Figure 2) (Kim et al., 2010; Kozłowski et al., 2016). Here we show that even under anoxic conditions the sole presence of NH_2OH also yields a large amount of N_2O (SI-S6), suggested as a new N_2O producing pathway by (cyt) P460 (Caranto et al., 2016). The addition of an electron donor like NO_2^- further increased N_2O production, highlighting the role of the primary N-substrates on N_2O dynamics, especially of NH_2OH .

The NDHA model captures the observed anoxic NH_2OH oxidation to N_2O with no HNO_2 production associated. A NH_2OH pulse in the concentration range of reported measurements (0.1 mgN/L) could be described in the calibration dataset (Figure 5, F-test = 1, $R^2 > 0.99$); and at higher NH_2OH concentrations (2 mgN/L) the model predicted the N_2O trend but not as accurately (Figure 5, F-test = 0, $R^2 = 0.97$). Anoxic NH_2OH oxidation to NO has been recently proposed by Caranto and Lancaster (2017) as the HAO-catalyzed reaction, where NO_2^- is produced only under aerobic conditions. The NO and NO_2^- producing reactions, together with the anoxic NO reduction to N_2O are captured by the NDHA model (SI-S10, Process 2, 3, 5). Based on the model structure

of other two-pathway models for AOB none can predict the observed N_2O dynamics. In certain models NH_2OH does not react under anoxic conditions (Ding et al., 2016; Pocquet et al., 2016), or reacts producing both N_2O and HNO_2 (Ni et al., 2014).

Under a variety of DO, HNO_2 and NH_3 concentrations the calibrated NDHA model could describe the observed N_2O dynamics. Other models, with varying degrees of complexity, have also described the effect of HNO_2 and DO (4-6 processes) but the effect of NH_2OH , the main driver of N_2O production, was not considered (Ding et al., 2016; Ni et al., 2014). The scenario analysis indicated a shift between the main pathway contributions governed by DO and HNO_2 (Figure 6). This relationship has been described by other two-pathway models, where ND was the main contributor to the N_2O emission factor during NH_4^+ oxidation and the highest N_2O emission factor was observed at low DO (Ni et al., 2014; Pocquet et al., 2016).

4.3. N_2O model calibration

4.3.1. Analysis of the NDHA parameter estimates.

In the last years new N_2O models have improved their best-fit predictions under different scenarios (i.e. varying DO, NO_2^-) by increasing the number of processes and variables considered. For example, all the models describe the ND pathway with a NO_2^- dependency (Ni et al., 2011; Pocquet et al., 2016; Schreiber et al., 2009), or the NN pathway as a fraction of the NH_2OH oxidation to NO_2^- (Ni et al., 2014; Pocquet et al., 2016). While the intermediates of NH_4^+ oxidation or the process rates are described differently, some parameters are common across N_2O models.

In this study, the contribution of the NN pathway ($\epsilon_{\text{AOB}} = 0.048\% \mu_{\text{AOB.HAO}}$) is in the low range of other reported values (0.052-0.15%), while the maximum N_2O production rate, described by $\eta_{\text{NOR}} = 0.16$, lies in the range (0.07 – 0.34). In agreement with the ND description of the model by Pocquet et al., (2016), the electron acceptor of the ND pathway was HNO_2 instead of NO_2^- . Increasing N_2O production rates were observed at higher HNO_2 but constant NO_2^- (9.5-10mgN/L, 0.8-1.5 $\mu\text{gHNO}_2\text{-N/L}$). The affinity for HNO_2 ($K_{\text{AOB.HNO}_2} = 0.67 \mu\text{gHNO}_2\text{-N/L}$, 17.1 mg NO_2^- -N/L, pH 7.5, 20 °C) could be estimated from experiments run at varying HNO_2 (0.16-1.5 $\mu\text{gHNO}_2\text{-N/L}$).

N/L). The affinity for NO_2^- is 100 times lower than other nitrifying systems, but 15 times higher than a NO_2^- -accumulating biomass ($K_{\text{AOB},\text{NO}_2^-} = 0.14, 282 \text{ mgN/L}$) (Pocquet et al., 2016; Schreiber et al., 2009). The difference could be explained by the operating conditions at which each biomass is acclimated: low NO_2^- for activated sludge systems ($\approx 0.5 \text{ mgN/L}$) and high NO_2^- for nitrifying reactors ($50\text{-}150 \text{ mg NO}_2^- \text{-N/L}$ in the parent reactor of this study). The NDHA model combined with the experimental design allows the simultaneous estimation of parameters describing main N-substrates and N_2O dynamics from simple respirometric experiments.

4.3.2. N_2O models: response validation and identifiability

As N_2O models produce better fits discrimination tools become more important. If the capabilities of two models to describe dynamic N_2O trends are similar (Lang et al., 2017; Pan et al., 2015) visual inspection or metrics such as R^2 are not sufficient, and more rigorous statistics as the F-test used in this study are necessary for model discrimination.

The parameter subset selection during calibration of each scenario was based on the lower AIC criteria. The identifiability of the estimated parameters was assessed by the correlation matrix and the precision of the estimated parameters ($\text{CV} < 5\%$). The triplet $K_{\text{AOB},\text{HNO}_2}$, $K_{\text{AOB},\text{NH}_2\text{OH},\text{ND}}$ and η_{NOR} were estimated with the same dataset, and based on their collinearity index ($\gamma > 15$) they are not identifiable and their values depend on the others. This metric is based on local sensitivities, and as shown in (Table 2), the high correlation between $K_{\text{AOB},\text{NH}_2\text{OH},\text{ND}}$ and η_{NOR} could be responsible for the high collinearity of the triplet. An improved experimental design or an additional dataset such as NO would improve the identifiability these parameters, as shown for other two-pathway N_2O models (Pocquet et al., 2016). Together with the best-fit prediction the 95% CI of the calibrated NDHA model bracketed the experimental datasets, validating the model response.

The uncertainty of estimates (CV, correlation matrix) is not always reported in literature (Ding et al., 2016; Kim et al., 2017; Spérandio et al., 2016), which hampers critical discrimination procedures. While some models have reported the estimated variance identifiability metrics are

scarce and not assessed. To the authors knowledge none of the proposed N₂O models has studied how the uncertainty of parameter estimates affects N₂O predictions (e.g. ARIL, PUCI). Moreover, the analysis of residuals shows that high frequency data such as online sensors are not totally discrete and can lead to autocorrelated residuals in N₂O measurements (SI-S5). When residuals are not independent not every point contains information for model validation, which leads to underestimation of the parameter uncertainty (CV << 0.001% (Peng et al., 2015)).

Hence, while very high precision of estimates is possible, testing the model response can avoid a possible over interpretation of the dataset. In this study we show that addressing parameter identifiability after model calibration will benefit N₂O model discrimination studies.

4.4. N₂O model uncertainty

With the final objective of designing N₂O mitigation strategies, the confidence of model predictions is critical when quantifying N₂O emissions. As a by-product of NH₄⁺ oxidation, the uncertainty associated to NH₄⁺ removal processes will propagate to N₂O predictions. The respirometric experimental design allowed for accurate estimates and narrow 95% confidence intervals for DO, NH₄⁺ and other N-species, which was critical to reduce the predicted uncertainty for N₂O (SI-S9). N₂O models have been calibrated by sequentially fitting the primary N-substrates followed by the N₂O dynamics (Ni et al., 2014; Pocquet et al., 2016). The effect of propagating the uncertainty from the calibration of primary N-substrates to N₂O was not discussed. Consequently, the precision of the N₂O calibration could be overestimated.

[Figure 7]

To evaluate the performance of the parameter estimation results N₂O emissions from a simulated case study were examined via Monte-Carlo simulations (pH = 7.5, NH₄⁺ = 70 mgN/L, DO = 0.3, 1.3 mg/L and NO₂⁻ = 0, 1, 5, 15, 100 mgN/L, 500 simulations). At pseudo-steady state the standard error of the Monte-Carlo simulations shows the propagated uncertainty. For the 10 parameters estimated in this study the normalized uncertainty associated to their default class (Sin et al.,

2009) is approximately 40% of the N₂O emission factor (Figure 7). If the calibration results from this experimental design are considered instead (Table 2), the uncertainty decreases to 10% (Figure 7). These results highlight the importance of considering uncertainty propagation in N₂O predictions, as N₂O emissions are greatly affected by the uncertainty of primary N-substrates.

Conclusions

- A novel experimental design to calibrate N₂O models through extant respirometry is proposed that combines DO and N₂O measurements.
- Parameters associated to NO₂⁻ and NH₄⁺ oxidation were sequentially fitted to DO consumption profiles by isolating individual processes. Five parameters were identified from the DO dataset and another five were estimated from the N₂O dataset with low uncertainty (CV < 10%).
- The NDHA model response was validated and described AOB-driven N₂O production at varying DO and HNO₂ concentrations.
- For the first time the uncertainty of the calibrated parameters was propagated to the model outputs in a simulation case study, and compared to the uncertainty from a reference case. The uncertainty of the N₂O emission factor predicted was reduced from ~ 40% of its value to ~ 10%.

Software availability

The MATLAB/SIMULINK code containing the implementation of the model is free upon request to the corresponding author.

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References

- Akaike, H., 1974. A new look at the statistical model identification. *IEEE Trans. Automat. Contr.* 19, 716–723. doi:10.1109/TAC.1974.1100705
- Beale, E.M.L., 1960. Confidence Regions in Non-Linear Estimation. *J. R. Stat. Soc. Ser. B-statistical Methodol.* 22, 41–88. doi:10.2307/2983877
- Bennett, N.D., Croke, B.F.W., Guariso, G., Guillaume, J.H.A., Hamilton, S.H., Jakeman, A.J., Marsili-Libelli, S., Newham, L.T.H., Norton, J.P., Perrin, C., Pierce, S.A., Robson, B., Seppelt, R., Voinov, A.A., Fath, B.D., Andreassian, V., 2013. Characterising performance of environmental models. *Environ. Model. Softw.* 40, 1–20. doi:10.1016/j.envsoft.2012.09.011
- Brockmann, D., Rosenwinkel, K.-H., Morgenroth, E., 2008. Practical identifiability of biokinetic parameters of a model describing two-step nitrification in biofilms. *Biotechnol. Bioeng.* 101, 497–514. doi:10.1002/bit.21932
- Brouwer, H., Klapwijk, A., Keesman, K.J., 1998. Identification of activated sludge and wastewater characteristics using respirometric batch-experiments. *Water Res.* 32, 1240–1254. doi:10.1016/S0043-1354(97)00334-5
- Brun, R., Kühni, M., Siegrist, H., Gujer, W., Reichert, P., 2002. Practical identifiability of ASM2d parameters--systematic selection and tuning of parameter subsets. *Water Res.* 36, 4113–27.
- Brun, R., Reichert, P., Kfinsch, H.R., 2001. Practical identifiability analysis of large environmental simulation models. *Water Resour. Res.* 37, 1015–1030.
- Campolongo, F., Saltelli, A., 1997. Sensitivity analysis of an environmental model: an application of different analysis methods. *Reliab. Eng. Syst. Saf.* 57, 49–69. doi:10.1016/S0951-8320(97)00021-5
- Caranto, J.D., Lancaster, K.M., 2017. Nitric oxide is an obligate bacterial nitrification intermediate produced by hydroxylamine oxidoreductase. *Proc. Natl. Acad. Sci.* 201704504. doi:10.1073/pnas.1704504114
- Caranto, J.D., Vilbert, A.C., Lancaster, K.M., 2016. Nitrosomonas europaea cytochrome P460 is a direct link between nitrification and nitrous oxide emission. *Proc. Natl. Acad. Sci.* 113, 14704–14709. doi:10.1073/pnas.1611051113
- Castro-Barros, C., Rodríguez-Caballero, A., Volcke, E.I.P., Pijuan, M., 2016. Effect of nitrite on the N₂O and NO production on the nitrification of low-strength ammonium wastewater. *Chem. Eng. J.* 287, 269–276. doi:10.1016/j.cej.2015.10.121
- Chandran, K., Hu, Z., Smets, B.F., 2008. A critical comparison of extant batch respirometric and substrate depletion assays for estimation of nitrification biokinetics. *Biotechnol. Bioeng.* 101, 62–72. doi:10.1002/bit.21871
- Chandran, K., Smets, B.F., 2005. Optimizing experimental design to estimate ammonia and nitrite oxidation biokinetic parameters from batch respirograms. *Water Res.* 39, 4969–78. doi:10.1016/j.watres.2005.10.001
- Cierkens, K., Plano, S., Benedetti, L., Weijers, S., de Jonge, J., Nopens, I., 2012. Impact of influent data frequency and model structure on the quality of WWTP model calibration and uncertainty. *Water Sci. Technol.* 65, 233–42. doi:10.2166/wst.2012.081
- Ciudad, G., Werner, A., Bornhardt, C., Muñoz, C., Antileo, C., 2006. Differential kinetics of ammonia- and nitrite-oxidizing bacteria: A simple kinetic study based on oxygen affinity and proton release during nitrification. *Process Biochem.* 41, 1764–1772. doi:10.1016/j.procbio.2006.03.032
- de Bruijn, P., van de Graaf, A.A., Jetten, M.S.M., Robertson, L.A., Kuenen, J.G., 1995. Growth of Nitrosomonas europaea on hydroxylamine. *FEMS Microbiol. Lett.* 125, 179–184.

- Ding, X., Zhao, J., Hu, B., Chen, Y., Ge, G., Li, X., Wang, S., Gao, K., Tian, X., 2016. Mathematical modeling of nitrous oxide production in an anaerobic/oxic/anoxic process. *Bioresour. Technol.* 222, 39–48. doi:10.1016/j.biortech.2016.09.092
- Dochain, D., Vanrolleghem, P.A., 2001. *Dynamic Modelling and Estimation in Wastewater Treatment Processes*. IWA Publishing, London, UK.
- Domingo-Félez, C., Pellicer-Nàcher, C., Petersen, M.S., Jensen, M.M., Plósz, B.G., Smets, B.F., 2017. Heterotrophs are key contributors to nitrous oxide production in activated sludge under low C-to-N ratios during nitrification-Batch experiments and modeling. *Biotechnol. Bioeng.* 114, 132–140. doi:10.1002/bit.26062
- Domingo-Félez, C., Smets, B.F., 2016. A consilience model to describe N₂O production during biological N removal. *Environ. Sci. Water Res. Technol.* 2, 923–930. doi:10.1039/C6EW00179C
- Ellis, T.G., Barbeau, D.S., Smets, B.F., Grady, C.P.L., 1996. Respirometric technique for determination of extant kinetic parameters describing biodegradation. *Water Environ. Res.* 68, 917–926. doi:10.2175/106143096X127929
- Gernaey, K., Petersen, B., Dochain, D., Vanrolleghem, P.A., 2002. Modeling aerobic carbon source degradation processes using titrimetric data and combined respirometric-titrimetric data: structural and practical identifiability. *Biotechnol. Bioeng.* 79, 754–67. doi:10.1002/bit.10337
- Graaf, A.A. Van De, Bruijn, P. De, Robertson, L.A., Jetten, M.S.M., Kuenen, J.G., 1996. Autotrophic growth of anaerobic ammonium-oxidizing microorganisms in a fluidized bed reactor. *Microbiology* 142, 2187–2196.
- Guo, L., Vanrolleghem, P. a., 2014. Calibration and validation of an activated sludge model for greenhouse gases no. 1 (ASMG1): prediction of temperature-dependent N₂O emission dynamics. *Bioprocess Biosyst. Eng.* 37, 151–163. doi:10.1007/s00449-013-0978-3
- Gustavsson, D.J.I., Tumlin, S., 2013. Carbon footprints of Scandinavian wastewater treatment plants. *Water Sci. Technol.* 68, 887. doi:10.2166/wst.2013.318
- Haefner, J.W., 2005. *Modeling Biological Systems: Principles and Applications*, Second. ed. Springer.
- Hiatt, W.C., Grady, C.P.L., 2008. An updated process model for carbon oxidation, nitrification, and denitrification. *Water Environ. Res.* 80, 2145–2156. doi:10.2175/106143008X304776
- Igarashi, N., Moriyama, H., Fujiwara, T., Fukumuri, Y., Tanaka, N., 1997. The structure of hydroxylamine oxidoreductase from a nitrifying chemoautotrophic bacterium, *Nitrosomonas europaea*. *Nature* 4, 276–284.
- Jin, X., Xu, C.-Y., Zhang, Q., Singh, V.P., 2010. Parameter and modeling uncertainty simulated by GLUE and a formal Bayesian method for a conceptual hydrological model. *J. Hydrol.* 383, 147–155. doi:10.1016/j.jhydrol.2009.12.028
- Kampschreur, M.J., van der Star, W.R.L., Wienders, H.A., Mulder, J.W., Jetten, M.S.M., van Loosdrecht, M.C.M., 2008. Dynamics of nitric oxide and nitrous oxide emission during full-scale reject water treatment. *Water Res.* 42, 812–26. doi:10.1016/j.watres.2007.08.022
- Kim, M., Wu, G., Yoo, C., 2017. Quantification of nitrous oxide (N₂O) emissions and soluble microbial product (SMP) production by a modified AOB-NOB-N₂O-SMP model. *Bioresour. Technol.* 227, 227–238. doi:10.1016/j.biortech.2016.11.127
- Kim, S.-W., Miyahara, M., Fushinobu, S., Wakagi, T., Shoun, H., 2010. Nitrous oxide emission from nitrifying activated sludge dependent on denitrification by ammonia-oxidizing bacteria. *Bioresour. Technol.* 101, 3958–63. doi:10.1016/j.biortech.2010.01.030
- Kozłowski, J.A., Kits, K.D., Stein, L.Y., 2016. Comparison of Nitrogen Oxide Metabolism among Diverse Ammonia-Oxidizing Bacteria. *Front. Microbiol.* 7, 1–9. doi:10.3389/fmicb.2016.01090

- Kozłowski, J. a, Price, J., Stein, L.Y., 2014. Revision of N₂O-Producing Pathways in the Ammonia-Oxidizing Bacterium *Nitrosomonas europaea* ATCC 19718. *Appl. Environ. Microbiol.* 80, 4930–4935. doi:10.1128/AEM.01061-14
- Lang, L., Pocquet, M., Ni, B.-J., Yuan, Z., Spérandio, M., 2017. Comparison of different two-pathway models for describing the combined effect of DO and nitrite on the nitrous oxide production by ammonia-oxidizing bacteria. *Water Sci. Technol.* 75, 491–500. doi:10.2166/wst.2016.389
- Law, Y., Lant, P., Yuan, Z., 2011. The effect of pH on N₂O production under aerobic conditions in a partial nitrification system. *Water Res.* 45, 5934–44. doi:10.1016/j.watres.2011.08.055
- Li, L., Xu, C.-Y., Xia, J., Engeland, K., Reggiani, P., 2011. Uncertainty estimates by Bayesian method with likelihood of AR (1) plus Normal model and AR (1) plus Multi-Normal model in different time-scales hydrological models. *J. Hydrol.* 406, 54–65. doi:10.1016/j.jhydrol.2011.05.052
- Lilliefors, H.W., 1967. On the Kolmogorov-Smirnov Test for Normality with Mean and Variance Unknown. *J. Am. Stat. Assoc.* 62, 399. doi:10.2307/2283970
- Machado, V.C., Tapia, G., Gabriel, D., Lafuente, J., Baeza, J.A., 2009. Systematic identifiability study based on the Fisher Information Matrix for reducing the number of parameters calibration of an activated sludge model. *Environ. Model. Softw.* 24, 1274–1284. doi:10.1016/j.envsoft.2009.05.001
- Magrí, A., Corominas, L., López, H., Campos, E., Balaguer, M., Colprim, J., Flotats, X., 2007. A Model for the Simulation of the SHARON Process: pH as a Key Factor. *Environ. Technol.* 28, 255–265. doi:10.1080/09593332808618791
- Ni, B.-J., Peng, L., Law, Y., Guo, J., Yuan, Z., 2014. Modeling of Nitrous Oxide Production by Autotrophic Ammonia-Oxidizing Bacteria with Multiple Production Pathways. *Environ. Sci. Technol.* 48, 3916–24. doi:10.1021/es405592h
- Ni, B.-J., Rusalleda, M., Pellicer-Nàcher, C., Smets, B.F., 2011. Modeling nitrous oxide production during biological nitrogen removal via nitrification and denitrification: extensions to the general ASM models. *Environ. Sci. Technol.* 45, 7768–76. doi:10.1021/es201489n
- Pan, Y., Ni, B.-J., Lu, H., Chandran, K., Richardson, D., Yuan, Z., 2015. Evaluating two concepts for the modelling of intermediates accumulation during biological denitrification in wastewater treatment. *Water Res.* 71, 21–31. doi:10.1016/j.watres.2014.12.029
- Park, H.-D., Noguera, D.R., 2007. Characterization of two ammonia-oxidizing bacteria isolated from reactors operated with low dissolved oxygen concentrations. *J. Appl. Microbiol.* 102, 1401–17. doi:10.1111/j.1365-2672.2006.03176.x
- Peng, L., Ni, B.-J., Ye, L., Yuan, Z., 2015. The combined effect of dissolved oxygen and nitrite on N₂O production by ammonia oxidizing bacteria in an enriched nitrifying sludge. *Water Res.* 73, 29–36. doi:10.1016/j.watres.2015.01.021
- Petersen, B., Gernaey, K. V., Vanrolleghem, P.A., 2001. Practical identifiability of model parameters by combined respirometric-titrimetric measurements. *Water Sci. Technol.* 43, 347–356.
- Pocquet, M., Wu, Z., Queinnec, I., Spérandio, M., 2016. A two pathway model for N₂O emissions by ammonium oxidizing bacteria supported by the NO/N₂O variation. *Water Res.* 88, 948–959. doi:10.1016/j.watres.2015.11.029
- Power, M., 1993. The predictive validation of ecological and environmental models. *Ecol. Modell.* 68, 33–50. doi:10.1016/0304-3800(93)90106-3
- Richardson, D., Felgate, H., Watmough, N., Thomson, A., Baggs, E., 2009. Mitigating release of the potent greenhouse gas N(2)O from the nitrogen cycle - could enzymic regulation

- hold the key? *Trends Biotechnol.* 27, 388–97. doi:10.1016/j.tibtech.2009.03.009
- Rodriguez-Caballero, A., Pijuan, M., 2013. N₂O and NO emissions from a partial nitrification sequencing batch reactor: exploring dynamics, sources and minimization mechanisms. *Water Res.* 47, 3131–40. doi:10.1016/j.watres.2013.03.019
- Sayavedra-Soto, L.A., Hommes, N.G., Russell, S.A., Arp, D.J., 1996. Induction of ammonia monooxygenase and hydroxylamine oxidoreductase mRNAs by ammonium in *Nitrosomonas europaea*. *Mol. Microbiol.* 20, 541–548. doi:10.1046/j.1365-2958.1996.5391062.x
- Schreiber, F., Loeffler, B., Polerecky, L., Kuypers, M.M., de Beer, D., 2009. Mechanisms of transient nitric oxide and nitrous oxide production in a complex biofilm. *ISME J.* 3, 1301–1313. doi:10.1038/ismej.2009.55
- Schreiber, F., Wunderlin, P., Udert, K.M., Wells, G.F., 2012. Nitric oxide and nitrous oxide turnover in natural and engineered microbial communities: biological pathways, chemical reactions, and novel technologies. *Front. Microbiol.* 3, 372. doi:10.3389/fmicb.2012.00372
- Sin, G., Gernaey, K. V., Neumann, M.B., van Loosdrecht, M.C.M., Gujer, W., 2009. Uncertainty analysis in WWTP model applications: a critical discussion using an example from design. *Water Res.* 43, 2894–906. doi:10.1016/j.watres.2009.03.048
- Soler-Jofra, A., Stevens, B., Hoekstra, M., Picioreanu, C., Sorokin, D., van Loosdrecht, M.C.M., Pérez, J., 2016. Importance of abiotic hydroxylamine conversion on nitrous oxide emissions during nitrification of reject water. *Chem. Eng. J.* 287, 720–726. doi:10.1016/j.cej.2015.11.073
- Spérandio, M., Pocquet, M., Guo, L., Ni, B.-J., Vanrolleghem, P.A., Yuan, Z., 2016. Evaluation of different nitrous oxide production models with four continuous long-term wastewater treatment process data series. *Bioprocess Biosyst. Eng.* 39, 493–510. doi:10.1007/s00449-015-1532-2
- Su, Q., Ma, C., Domingo-Félez, C., Kiil, A.S., Thamdrup, B., Jensen, M.M., Smets, B.F., 2017. Low nitrous oxide production through nitrifier-denitrification in intermittent-feed high-rate nitrification reactors. *Water Res.* 123, 429–438. doi:10.1016/j.watres.2017.06.067
- Terada, A., Lackner, S., Kristensen, K., Smets, B.F., 2010. Inoculum effects on community composition and nitrification performance of autotrophic nitrifying biofilm reactors with counter-diffusion geometry. *Environ. Microbiol.* 12, 2858–2872.
- Yu, R., Kampschreur, M.J., van Loosdrecht, M.C.M., Chandran, K., 2010. Mechanisms and specific directionality of autotrophic nitrous oxide and nitric oxide generation during transient anoxia. *Environ. Sci. Technol.* 44, 1313–9. doi:10.1021/es902794a

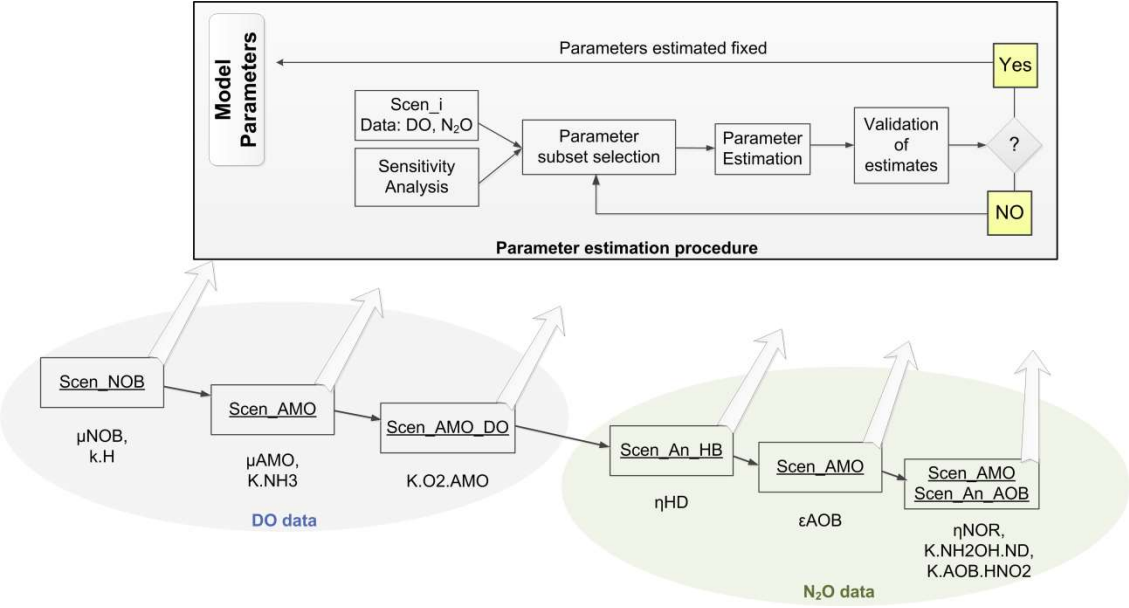


Figure 1 – Schematic of the parameter estimation procedure.

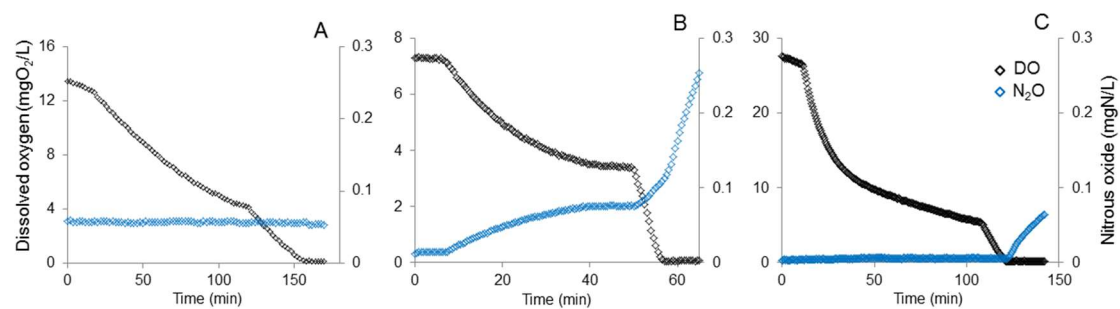


Figure 2 – Dissolved oxygen and liquid nitrous oxide concentrations during experiments: Scen_NOB (NO₂⁻ pulses) (A), Scen_HAO (NH₂OH pulses) (B) and Scen_AMO (NH₄⁺ pulses) (C) (3-4.5 mgN/L).

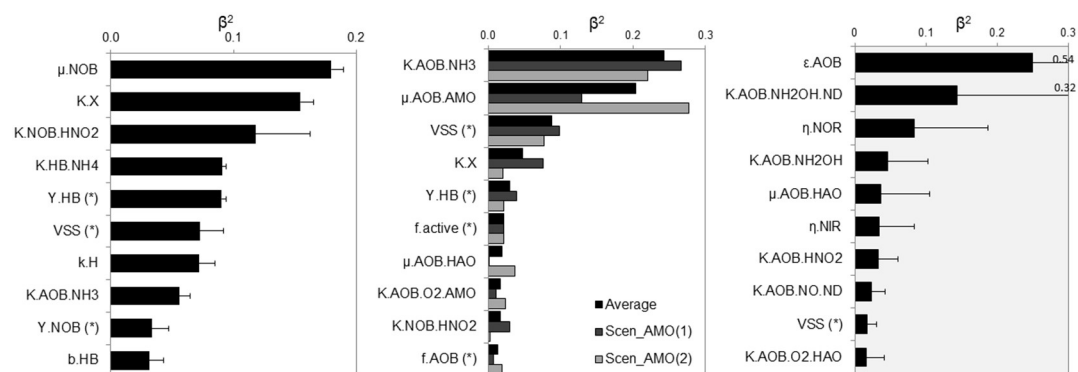


Figure 3 – Parameter sensitivity ranking of scenarios used during calibration for DO (white background) and N₂O (grey background). Scen_NOB (left), Scen_AMO (middle and right). (*) Parameters not considered for calibration. The error bars correspond to the standard deviation between experiments of the same scenario.

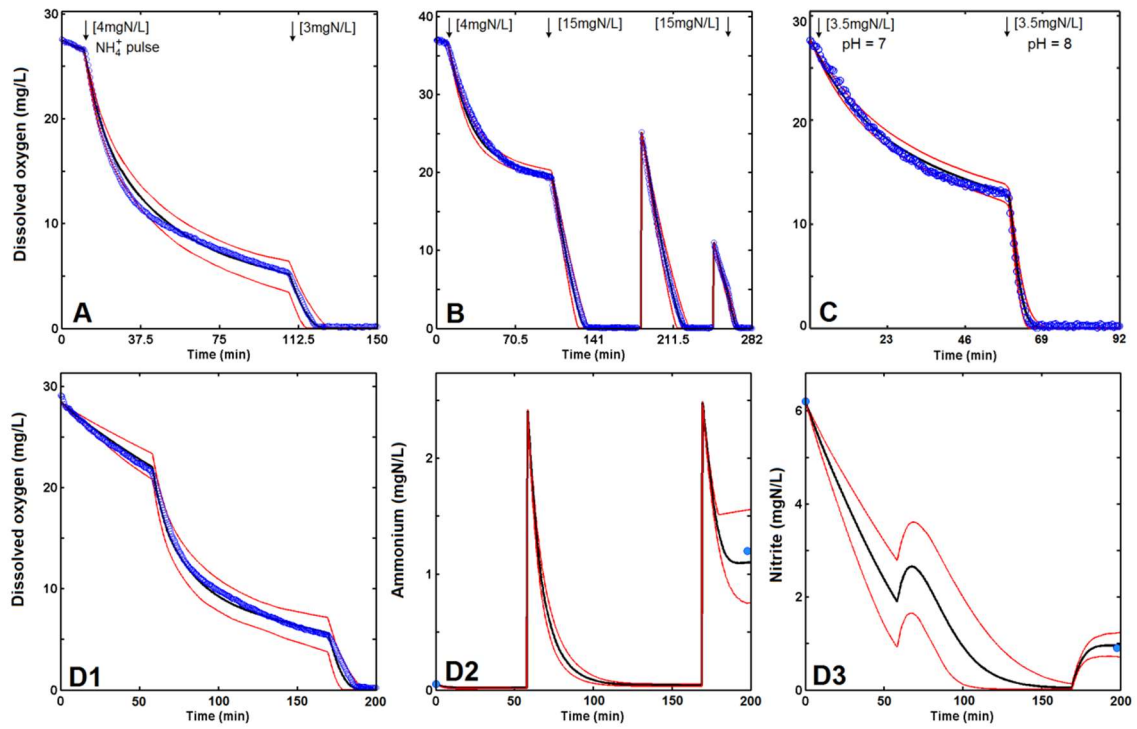


Figure 4 – Experimental DO, NH_4^+ and NO_2^- (blue markers) and model predictions (black line best-fit, red lines 95% CI). Datasets from Scen_AMO, calibration: A and B; validation: C and D.

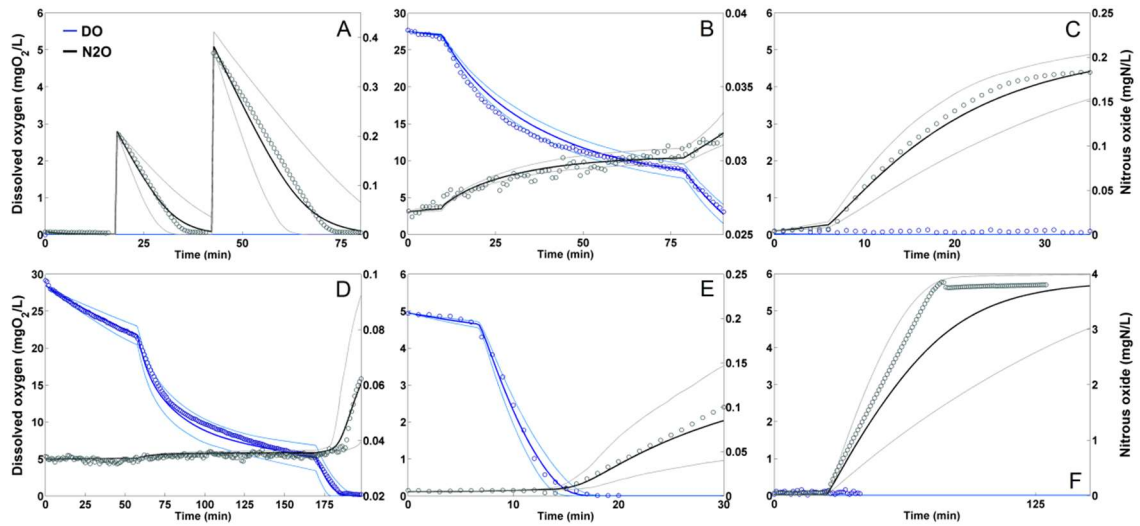


Figure 5 – Experimental N₂O (black) and DO (blue), and model predictions (dark line best-fit, light lines 95% CI) for the N₂O calibration. Datasets from calibration: (A) Scen_An_HB (N₂O pulse), (B) Scen_AMO (Aerobic NH₄⁺ pulse), (C) Scen_An_AOB (Anoxic NH₂OH pulse). Datasets from validation: (D, E) Scen_AMO (Aerobic → anoxic NH₄⁺ pulse), (F) Scen_An_AOB (NH₂OH pulse).

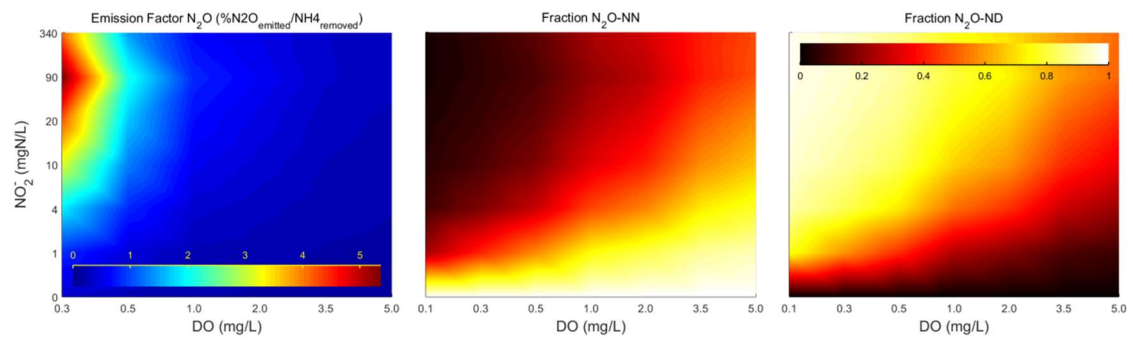
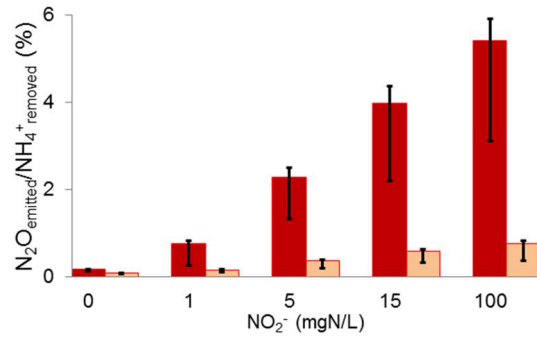


Figure 6 – Scenario analysis using the validated NHDA simulation model. Simulations were run under constant DO levels (0.1 - 0.3 - 0.5 - 1.0 - 2.0 - 3.5 - 5.0 mg/L), NO_2^- (0 – 1 – 4 – 10 – 20 – 90 – 340 mgN/L). (Left) N_2O emission factor (% $\text{N}_2\text{O}/\text{NH}_4^+$), colorbar: 0 – 5%, blue - red. (Middle, Right) NN, ND Pathway contribution (-), colorbar: 0 – 1, black - white.



| Parameters | Normalized variance | Uncertainty |
|------------------------------|---------------------------------|-------------------------|
| All | <div style="width: 61%;"></div> | 61% Reference class |
| | <div style="width: 40%;"></div> | 40% Estimated (Table 2) |
| Subset estimated (10 par) | <div style="width: 44%;"></div> | 44% Reference class |
| | <div style="width: 8%;"></div> | 8% Estimated (Table 2) |

Figure 7 – Model evaluation results (500 runs) at pseudo-steady state for NH_4^+ removal at constant DO and NO_2^- : AOB-enriched biomass, $\text{pH} = 7.5$, $\text{NH}_4^+ = 70$ mgN/L, DO $[0.3 - 1.3]$ mg/L and $\text{NO}_2^- [0 - 1 - 5 - 15 - 100]$ mgN/L. (Top) N_2O emission factor at low DO (red) and high DO (light red); top standard error corresponds to uncertainty of estimated parameters only (Table 2), bottom standard error corresponds to uncertainty in All model parameters. (Bottom) Normalized variance for uncertainty considered in All model parameters (dark grey) or only for the 10 parameters estimated in this study (light grey) (Table 2) with default uncertainty (top bar) or from this study (Table 2) (bottom bar).